

CLONE OF THE GENE OF THE DENSE GRANULE ANTIGEN (GRA6) OF THE *TOXOPLASMA GONDII* PIG STRAIN

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Abstract. The gene of GRA6 of *Toxoplasma gondii* pig strain was cloned; and the value of recombinant GRA6 as a diagnostic antigen of toxoplasmosis in China was investigated. A pair of primers was synthesized according to the DNA sequence of GRA6 of *T. gondii* RH strain, and the DNA of pig strain tachyzoites was prepared. A single, specific DNA fragment was successfully amplified from the genomic DNA of tachyzoites of *T. gondii*. This gene fragment was cloned into the plasmid pUC19 to form the recombinant. The recombinant plasmid was purified and the foreign DNA fragment was sequenced. The result of DNA sequencing showed that the open reading frame of GRA6 is composed of 693 base pairs and encodes 230 amino acids and is 100% homologous with the sequence of GRA6 of *T. gondii* RH strain.

INTRODUCTION

Toxoplasma gondii is a worldwide parasitic protozoan with a large variety of hosts, which provide a source of human infection. Although the majority of toxoplasma infections are asymptomatic, reactivation may lead to severe disease in immunodeficient or post-transplant patients and may cause abortion or congenital malformation in pregnant women (Luft and Remington, 1992; Israelski and Remington, 1993; Remington *et al*, 1995).

The majority of the tests presently used for toxoplasmosis diagnosis are serological assays, although they give satisfying results, accurate differentiation between recently acquired and chronic toxoplasmosis remains problematic; false positive reactions with antinuclear factor, rheumatoid factors, or naturally-occurring human antibodies and false-negative reactivity due to competitive inhibition by high levels of specific IgG antibodies have been described (Araujo *et al*, 1971; Konishi, 1991). The presence of specific IgM antibodies is not always indicative of an acute infection with

T. gondii, since IgM antibodies can persist for months or years; moreover, different techniques used for antigen extraction result in a wide variation of different antigens in different preparations and in a lack of qualitative and quantitative agreement between different diagnostic tests. The use of recombinant antigen could avoid these drawbacks and permit the development of improved diagnostic tests. In this study, the gene of GRA6 of *T. gondii* pig strain was cloned by PCR and sequenced; expressing this molecule *in vitro* and using it as a standard antigen would aid the diagnosis of toxoplasmosis in China.

MATERIALS AND METHODS

Reagents

Wizard Plus Minipreps DNA Purification System, Wizard PCR Preps DNA Purification and Wizard DNA Clean up DNA Purification System were purchased from the Promega Company.

Toxoplasma gondii pig strain

The stain was isolated from oral mucosa of a pig. The tachyzoites of *T. gondii* pig strain were collected from the ascites of a mouse that had been inoculated three days earlier.

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Extraction of DNA

Fresh tachyzoites were broken by SDS, the genomic DNA was extracted by phenol: chloroform and deposited by cooled ethanol at -20°C for 30 minutes. After being centrifuged at 12000g for 10 minutes, the DNA was dissolved with 50 µl double distilled water (ddH₂O).

Primers

A pair of primers, P1 and P2, was designed according to the DNA sequence of the GRA6 of RH strain:

P1: 5'-TTGGATCCATGGCACACGGTGGC-3'
EcoRI

P2: 5'-GCGGATCCTTAATAATCAAACAC-3'
EcoRI

The restrictive site of EcoRI was added at the 5'-ends of primer P1 and P2. The primers were synthesized by the Shanghai Sangon Biological Engineering Co.

Polymerase chain reaction (PCR)

P1 (12.5 µM) 2 µl; P2 (12.5 µM) 2 µl; 10x*Taq* polymerase buffer 10 µl; MgCl₂ (25 mM), 10 µl; 4dNTP (2.0 mM), 10 µl; template DNA, 2 µl; *Taq* DNA polymerase, 1.5 µl (4U), were mixed, then 62.5 µl of ddH₂O were added to a final volume of 100 µl. PCR was performed: first at 95°C for 9 minutes; second, at 94°C, 30 seconds; and then at 55°C for 30 seconds and 72°C for 30 seconds for 35 cycles, followed by 72°C for 7 minutes. After PCR, 10 µl of the products were taken and analyzed by agarose electrophoresis. 1 µl of T4 DNA polymerase (3U) was added to 80 µl of products and incubated at 37°C for 15 minutes.

Purification of the target DNA fragment

The PCR product was a single DNA band on agarose electrophoresis, with a molecular weight of approximately 700 bp, similar to the predicted GRA6 gene fragment. The target DNA fragment was purified by a direct purification method using the Wizard PCR Preps Purifi-

cation System (Promega) according to the manufacturer's instructions.

Cloning of the target DNA fragment

Digestion: the purified target DNA fragment and plasmid vector pUC19 were digested with restriction enzymes EcoRI. The products were purified with the Wizard DNA Clean Up System (Promega) according to the manufacturer's instructions.

Ligation: 6 µl (100 ng) of the purified and digested target DNA, 4 µl (40 ng) of plasmid vector pUC19, and 1 µl of 10xligation buffer and 1 µl (3U) of T4 ligase (Promega) were placed in a 0.5ml sterile microcentrifuge tube, mixed and incubated at 14°C for 12-14 hours.

Transformation: the competent cells of *E. coli* JM109 were prepared according to the method of Sambrook *et al* (1989) and kept at 4°C for 24 hours. 200 µl of competent JM109 cells and 10 µl of ligated mixture were mixed in a 0.5 ml sterile tube, placed on ice for 30 minutes, and then heat shocked at 42°C for 90 seconds. 1 ml of pre-warmed LB medium at 37°C was added to the tube and incubated with shaking at 37°C for 45 minutes. 200 µl of incubated product was inoculated on a LB culture plate with 50 µg/ml of ampicillin, and incubated at 37°C overnight.

Identification of the positive clones

The plasmid DNA contained in the single colony was extracted by the fast plasmid-size identification method (Sambrook *et al*, 1989). The size of the recombinant DNA was determined by agarose electrophoresis. The DNA of recombinant plasmid in a single colony was purified with the Wizard Minipreps DNA Purification System (Promega). 2 µl of purified recombinant plasmid DNA were amplified by PCR with the specific primers, P1 and P2, and the products of PCR were identified by electrophoresis.

Analysis of the DNA sequence

The purified recombinant DNA was se-

quenced at the Jiangsu Institute of Parasitic Diseases and at the Shanghai Sangon Biological Engineering Co. The DNA sequences were analyzed using the computer software of Wdnasis 2.5.

Analysis of the deduced amino acid sequence

The amino acid sequence was deduced according to the DNA sequence of TPI of pig strain and analyzed using the computer software of Prosis.

RESULTS

Target DNA fragment

A single, approximately 700 bp, specific fragment was amplified from DNA of the tachyzoites of pig strain by PCR using primers P1 and P2 (Fig 1).

Cloning of target DNA and analysis of recombinant DNA with PCR

The purified, end-repaired target DNA fragment was digested with restrictive enzyme EcoR1, and inserted into the vector pUC19 at the EcoR1 site. The recombinant shown in Fig 2, containing target DNA and pUC19, was produced. The plasmid DNA of the recombinant was purified and amplified by PCR with primers P1 and P2 again, after which a single DNA, specific fragment (approximately 700 bp) was produced that was identical to the target DNA fragment produced from genomic DNA by PCR.

DNA sequence of the target DNA fragment

The purified DNA of the recombinant was sequenced. The result showed that the length of the open reading frame of GRA6 was 693 bp, which is the same as the GRA6 of RH standard strain reported by Fazaeli *et al*, 2000 and had 99% homology to the GRA6 of Castell's strain. This indicated that the target DNA fragment should be GRA6 of *T. gondii* pig strain. The base composition of GRA6 was: A: 166; T: 146; G: 225; C: 155; A+T/G+C: 0.82:1, GC: 54.91% (Fig 3).

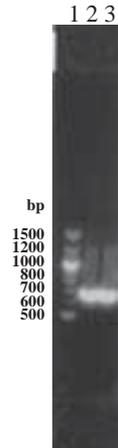


Fig 1–The target fragment amplified DNA.
Lane 1: DNA marker
Lanes 2 and 3: PCR band of GRA6

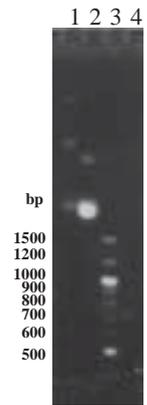


Fig 2–The recombinant (GRA6+pUC19) and PCR product.
Lane 1: recombinant (GRA6+pUC19)
Lane 2: plasmid pUC19
Lane 3: DNA marker
Lane 4: the PCR product from the recombinant plasmid DNA

Analysis of the amino acid sequence

The deduced amino acid sequence according to the open reading frame of the encoding GRA6 DNA sequence included 230 amino acids (Fig 3). The expected molecular weight of the molecule was 23,973.02. Its isoelectric point

1	<u>ATGGCACACGGTGGCATCCATCTGAGGCAGAAGCGTAACTTCTGTCCCTTAACTGTCTCC</u>	60
1	M A H G G I H L R Q K R N F C P L T V S	20
61	ACAGTTGCTGTGGTCTTTGTAGTCTTCATGGGTGACTCGTCAATTCGTGGGTGGAGTC	120
21	T V A V V F V V F M G V L V N S L G G V	40
121	GCTGTCGCAGCAGACAGCGATGGTGTAAAGCAGACCCCTTCGGAACCGGTTCCGAGCGGT	180
41	A V A A D S D G V K Q T P S E T G S S G	60
181	GGACAGCAAGAAGCAGTGGGGACCACTGAAGACTATGTCAACTCTTCGGCGATGGGCGGT	240
61	G Q Q E A V G T T E D Y V N S S A M G G	80
241	GGCCAAGCGACTCGTTAGCTGAAGATGATAACAACCTCCGATGCGGCGGAGGGCGACGTT	300
81	G Q G D S L A E D D T T S D A A E G D V	100
301	GACCCTTTTCCCGTGTGGCGAATGAGGGGAAGTCGGAGGCGCGTGGCCCGTCCGCTCGAG	360
101	D P F P V L A N E G K S E A R G P S L E	120
361	GAAAGAATCGAAGAACAGGGCACAGACGACGTTACTCCTCTGTTCAGAACCACAAGCG	420
121	E R I E E Q G T R R R Y S S V Q E P Q A	140
421	AAGGTGCCTAGCAAACGAACACAGAAACGCCACAGACTCATTGGTGCTGTGGTGTGGCA	480
141	K V P S K R T Q K R H R L I G A V V L A	160
481	GTATCTGTGGCAATGCTTACCGCTTCTTCTTCGAAGGACTGGACGACGCTCTCCCCCA	540
161	V S V A M L T A F F L R R T G R R S P P	180
541	GAACCATCTGGGGATGGTGGTGGAAATGATGCAGGCAATAATGCTGGGAACGGTGGGAAT	600
181	E P S G D G G G N D A G N N A G N G G N	200
601	GAAGGCAGAGGTTACAGAGGCAGAGGTGAAGGAGGAGGCGAGGATGACAGGCGCGCTTG	660
201	E G R G Y R G R G E G G G E D D R R A L	220
661	CACCCGGGAAGTGTGAATGTGTTTATTATTA <u>A</u>	693
221	H P G S V N V F D Y *	231

Fig 3—Nucleotide sequence with amino acid tachyzoites translations of GRA6. Predicted initiation and termination codons are underline.

(pI) was 5.36, which relates to acidic proteins. The amino acid composition is shown in Fig 4.

DISCUSSION

In recent years, the techniques of molecular biology, *eg* PCR and dot blot analysis, have been applied to the detection of *T. gondii* DNA in clinical samples. However, serological tests are the basic methods of screening and for determining the phase of infection. Most serological tests for toxoplasma require the preparation of parasite antigens from tachyzoites harvested from mice or cell culture systems.

However, the use of whole tachyzoite antigens can result in false-positive reactions and, therefore, recombinant antigens have recently been developed, which are less expensive and easier to standardize in IgG and IgM serological tests.

In this study, we amplified the DNA fragment of the open reading frame of GRA6 from the DNA of the tachyzoites of *T. gondii* pig strain with primers P1 and P2 by PCR. The results of the DNA sequence analysis of GRA6 showed that the length of the open reading frame (ORF) is 693 bp. This DNA fragment has 100% homology to the GRA6 of pig strain reported by Fazaeli *et al* (2000) has

GRA6 GENE OF *T. GONDII*

Amino acid		Mol%	Count (CNT)	Residue weight (RW)	CNT * RW
Gly	G	15.65	36	57.05	2,053.80
Ala	A	8.70	20	71.07	1,421.40
Val	V	10.00	23	99.13	2,279.99
Leu	L	5.22	12	113.15	1,357.80
Ile	I	1.30	3	113.15	339.45
Ser	S	8.26	19	87.07	1,654.33
Thr	T	5.22	12	101.10	1,213.20
Cys	C	0.43	1	103.14	103.14
Met	M	1.74	4	131.19	524.76
Asp	D	6.09	14	115.08	1,611.12
Asn	N	4.35	10	114.10	1,141.00
Glu	E	6.96	16	129.11	2,065.76
Gln	Q	3.91	9	128.13	1,153.17
Arg	R	8.26	19	156.18	2,967.42
Lys	K	2.61	6	128.17	769.02
His	H	1.74	4	137.14	548.56
Phe	F	3.04	7	147.17	1,030.19
Tyr	Y	1.74	4	163.17	652.68
Trp	W	0.00	0	186.20	0.00
Pro	P	4.78	11	97.11	1,068.21
Asx	B	0.00	0	114.59	0.00
Glx	Z	0.00	0	128.62	0.00
***	*	0.00	0	0.00	0.00
Xxx	X	0.00	0	118.88	0.00
Total residue wieght --->					23,955.00
					+) 18.02
Molecular weight --->					23,973.02

Fig 4–The amino acid composition of the deduced GRA6.

99% homology to the GRA6 of Castell’s strain, which indicated that the DNA fragment that we produced is the gene of GRA6 molecule of the pig strain.

The GRA6 gene is a single-copy gene (Lecordier *et al*, 1995); as the mRNA is readily decomposed, it is difficult to clone this gene from a mRNA or a cDNA library, so we amplified it from the genomic DNA by PCR directly; the sequence of the ORF of GRA6 does not contain an intron sequence and the technique proved successful.

GRA6 is detected in the dense granules of tachyzoites, and in parasitophorous vacuoles, and secreted from the parasitophorous vacuoles. The GRA6 operates as an antigenic stimulus only during host cell invasion and during

the phase of tachyzoite division: anti-GRA6 antibodies might be shaped at an early point during the immune response. The value of granule antigen GRA6 fusion protein (Redlich and Muller, 1998) as a diagnostic antigen has been established.

We will, in the future, express the GRA6 of pig strain *in vitro* and investigate its value as a diagnostic antigen in China.

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